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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 5/00, 14/00, A61K 38/00, A01N 43/04

A1

(11) International Publication Number: WO 98/35981

(43) International Publication Date: 20 August 1998 (20.08.98)

(21) International Application Number: PCT/US98/02753

(22) International Filing Date: 13 February 1998 (13.02.98)

(30) Priority Data:
60/038,375 13 February 1997 (13.02.97) US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PREVENTION AND TREATMENT OF HEPATOCELLULAR CANCER

(57) Abstract

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*(Referred to in PCT Gazette No. 45/1998, Section II)

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PREVENTION AND TREATMENT OF HEPATOCELLULAR CANCER

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of co-pending United States Patent Application Serial Number 60/038,375, entitled METHOD AND COMPOSITIONS FOR PREVENTING AND TREATING HEPATOCELLULAR CANCER, filed February 13, 1997.

BACKGROUND

Primary liver cancer is a major cause of cancer deaths worldwide. Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, having an incidence of approximately 1.2 million cases per year. In some areas of the world, such as Southeast Asia and South Africa, hepatocellular carcinoma is one of the most common types of malignancies. The high frequency of the diseases appears to be related to the high incidence of hepatitis in these regions.

Curative therapy of hepatocellular carcinoma is currently limited to individuals with nonmetastatic disease and involves surgical resection of the tumor with or without liver transplantation. Even surgical resection and transplantation, however, do not cure most tumors because of recurrence after resection. Chemotherapeutic approaches to treatment have, to date, been largely ineffective. There have been no significant advances in the treatment of hepatocellular carcinoma during the last two decades.

Therefore, there remains a need for an effective treatment for hepatocellular carcinoma. The treatment should ideally be suitable for use in lesser developed countries that have the highest incidence of the disease. Further, the treatment should be appropriate for use in individuals with unresectable tumors and with metastatic disease.

SUMMARY

According to one embodiment of the present invention, there is provided a method for preventing or for treating cancer, such as hepatocellular carcinoma, in a mammal, including a human. The method comprises the step of creating an immune response in the mammal to at least part of the ammino acid sequence of an alphafetoprotein molecule.

The step of creating an immune response can comprise administering to the mammal at least one composition including a peptide comprising at least part of the alphafetoprotein amino acid sequence or at least one composition including a peptide

comprising at least part of the alphafetoprotein amino acid sequence with at least one amino acid substitution. The step of creating an immune response can also comprise administering to the mammal at least one composition including at least part of the cDNA sequence for the alphafetoprotein molecule. Further, the step of creating an immune response can comprise administering to the mammal at least one composition including immune system cells transduced with a recombinant vector that expresses alphafetoprotein cDNA.

According to another embodiment of the present invention, there is provided a composition for immunizing a human to prevent or to treat cancer. The composition can comprise a peptide selected from the group consisting of AFP5, AFP7, AFP13, AFP14, AFP18, AFP22, AFP23, AFP28 AFP38, AFP39, AFP45, AFP49, SEQ ID NO:2 and SEQ ID NO:3.

FIGURES

These and other features, aspects and advantages of the present invention will become better understood with regard to the following description, appended claims, and accompanying figures where:

Figure 1 is a bar graph showing the relative cytotoxicity of CTL generated using human AFP49 peptide;

Figure 2 is a bar graph showing the percent specific lysis versus targets for a standard chromium release assay of CTL generated from peptide-pulsed PBMC from a normal, HLA A2.1 donor, assayed against both peptide targets and AFP targets;

Figure 3 is a plot of mean tumor volume versus days after a tumor challenge of BWIC3, a mAFP-positive murine tumor cell line, for mice immunized with murine AFP cDNA (open boxes), and mice that were not immunized (closed squares);

Figure 4 is a plot of mean tumor volume versus days after a tumor challenge of EL4(parental), a non mAFP-producing murine tumor cell line, for mice immunized with murine AFP cDNA (open boxes) and mice that were not immunized (closed circles);

Figure 5 is a plot of mean tumor volume versus days after a tumor challenge of EL4(AFP), an mAFP-producing murine tumor cell line, for mice immunized with murine AFP cDNA (open boxes), and mice that were not immunized (closed circles);

Figure 6 is a plot of average tumor diameter versus days after a tumor challenge of FSA C3H background fibrosarcoma cells stably transfected with AFP-expressing vector or FSA C3H background fibrosarcoma cells stably transfected with the neo-expressing

vector only, for mice immunized with plasmid DNA using mouse AFP-AdVShuttle vector neo-containing expression plasmids (closed circles, lower closed squares, closed diamonds) and mice that were not immunized (upper closed squares, closed inverted triangles);

Figure 7 is a plot of average tumor diameter versus days after a tumor challenge of BWIC3 for mice immunized with a plasmid vector that synthesized the mouse AFP gene (closed circles) and mice that were not immunized (closed squares);

Figure 8 is a plot of average tumor diameter versus days after a tumor challenge of BWIC3 for mice that were immunized with a plasmid vector that synthesized the mouse AFP gene (closed triangles, closed diamonds, closed inverted triangles) and mice that were not immunized (closed circles and closed squares);

Figure 9 is an RT-PCR analysis of mRNA isolated from murine DC transduced with AdVmAFP at various multiplicities of infection (MOI), lanes 4-7, compared with various controls, lanes 2, 3, 8 and 9;

Figure 10 is a plot of mean tumor volume versus days after a tumor challenge of EL4(AFP), an mAFP-producing murine tumor cell line, for mice immunized with AdVmAFP transduced dendritic cells (closed square), mice that were immunized with various control substances (closed upright triangles) and closed inverted triangles) and mice that were not immunized (closed circle); and

Figure 11 is a plot of mean tumor volume versus days after a tumor challenge of BWIC3, an mAFP-producing murine tumor cell line, for mice immunized with AdVmAFP transduced dendritic cells (closed squares) and mice that were not immunized (closed circles).

DESCRIPTION

According to one embodiment of the present invention, there is provided a method for preventing or for treating cancer, including hepatocellular carcinoma, in a mammal such as a human where the cancer bears at least a portion of the alphafetoprotein molecule on its surface, by creating an immune response in the mammal to at least part of the alphafetoprotein molecule. The method involves immunizing or genetically manipulating the mammal having the cancer to produce an immune response to at least part of the alphafetoprotein molecule present on the surface of the cancer cells. Then, the immune system of the affected mammal is allowed to destroy the cancer cells bearing the surface marker, thereby preventing a clinical cancer or treating an established cancer.

The majority of human hepatocellular carcinoma cells synthesize human alphafetoprotein (hAFP), a 609 amino acid residue protein, SEQ ID NO:1, which is normally produced by fetal liver cells up until about the time of birth. Hepatocellular carcinoma cells tend to display at least part of the alphafetoprotein molecule on their surface. The presence of alphafetoprotein in hepatocellular carcinoma has been used a marker for screening and diagnostic purposes.

Because alphafetoprotein is normally present during the development of the immune system, it would naturally be assumed that the immune system would not retain the capacity to respond immunologically to the protein. One aspect of the present invention involves the discovery that the immune system of a mammal can be made to respond to alphafetoprotein as a foreign protein and to react to cells having at least part of the alphafetoprotein molecule on its surface as foreign cells. Generating this immune response can, therefore, be used to prevent hepatocellular carcinoma and to treat the disease by causing the mammal's immune system to destroy hepatocellular carcinoma cells.

As disclosed herein, immunization to alphafetoprotein can be accomplished by a variety of means including immunization with synthetic peptides comprising at least part of the alphafetoprotein sequence including synthetic peptides based on at least part of the alphafetoprotein sequence, but have substitutions or other alterations, immunization with at least part of the cDNA sequence for alphafetoprotein thereby causing production and presentation of at least part of the alphafetoprotein molecule to the appropriate immune system cells, the introduction of genetically engineered antigen presenting cells into the mammal, and the use of gene therapy viral vectors to cause the expression of at least part of the alphafetoprotein molecule. The goal of this immunization is to activate alphafetoprotein peptide specific T lymphocytes to create the immune response against cells bearing these surface markers, and preferably thereby to activate cytotoxic T lymphocytes to destroy hepatocellular carcinoma cells.

1) DETERMINATION AND PRODUCTION OF HUMAN ALPHAFETOPROTEIN PEPTIDES THAT CREATE AN IMMUNE RESPONSE IN HUMANS

In order to determine if any portion of the human alphafetoprotein molecule is capable of creating an immune response in humans, a series of peptides derived from the whole human alphafetoprotein (hAFP) molecule, SEQ ID NO:1, were tested to determine whether, as class I-restricted peptides, they were capable of generating antitumor responses

and capable of serving as target molecules for cytotoxic lymphocytes (CTL). Potentially immunogenic peptides derived from hAFP were selected on the basis of their potential conformity to the HLA A2.1 class I binding grove. HLA A2.1 (HLA A*0201 in the World Health Organization subtype nomenclature) was chosen because it is the most common allele among Caucasians and is also well distributed among other populations. The determination was made as follows.

First, peptide sequences from hAFP, SEQ ID NO:1, were identified that would potentially bind to HLA A2.1 according to published consensus sequences. HLA A2.1 is believed to bind peptides that are eight to ten amino acids in length, but preferably peptides that are nine mers. Amino acids isoleucine, leucine and methionine are believed to be important anchor residues in peptide position 2 and amino acids isoleucine, leucine and valine are believed to be important anchor residues in peptide positions 9 or 10, depending on the peptide length.

Appropriate peptide sequences that conformed to the HLA A2.1 class I binding motif were identified using the University of Wisconsin Genetics Computer Group Program "find patterns" to screen the hAFP sequence, SEQ ID NO:1, and identify nine and ten mer peptides that contained two strong binding "anchor" residues, one at position 2 and one at position 9 or 10 for peptides having nine and ten mers, respectively (designated "strong" peptides); only one strong binding anchor residue (designated "intermediate" peptides); or no strong binding anchor residue (designated "weak" peptides), but having other positive binding residues. Peptide sequences that contained more than one residue thought to abolish binding were eliminated.

The screening study identified a total of seventy-two peptide sequences that potentially conformed to the HLA A2.1 class I binding motif but six of these sequences were eliminated from further consideration because they were difficult to synthesize due to their high hydrophobicity. The remaining sixty-six peptide sequences were synthesized for testing by Chiron Mimetopes (Victoria, Australia) according to techniques known to those with skill in the art. These included ten "strong" peptide sequences, forty-three "intermediate" peptide sequences and thirteen "weak" peptide sequences. Referring now to Table I, there is shown from right to left, respectively, the peptide designation number, the residues of the hAFP sequence, SEQ ID NO:1, represented by the peptide sequence and the amino acids the peptide comprises for each of the sixty-six peptide sequences. The peptide designation

number is based on the order of receipt of the peptide from Chiron and is, therefore, nonsequential with

TABLE I

HUMAN AFP PEPTIDE SEQUENCES

Peptide Designation Name	Residues in hAFP Protein	Amino Acid Sequence
AFP1	449-457	AITRKMAAT
AFP2	434-442	AYTKKAPQL
AFP3	218-226	LLNQHACAV
AFP4	257-265	KLVLDVAHV
AFP5	158-166	FMNKFIYEI
AFP6	135-143	SIPLFQVPE
AFP7	12-20	LLNFTESRT
AFP8	54-62	FVQEATYKE
AFP9	58-66	ATYKEVSKM
AFP10	61-69	KEVSKMVKD
AFP11	121-129	RHNCFLAHK
AFP12	456-464	ATAATCCQL
AFP13	404-412	YIQESQALA
AFP14	441-450	QLTSSELMAI
AFP15	242-250	KLSQKFTKV
AFP16	211-219	KELRESSLL
AFP17	514-522	SLVVDETYV
AFP18	178-186	ILLWAARYD
AFP19	187-195	KIIPSCCKA
AFP20	270-278	CRGDVLDCL
AFP21	291-299	QQDTLSNKI
AFP22	547-556	TMKQEFLINL
AFP23	555-563	NLVKQKPQI
AFP24	570-578	AVIADFSGL
AFP25	469-477	LLACGEGAA
AFP26	470-478	LACGEGAAD
AFP27	438-447	KAPQLTSSEL
AFP28	287-295	YICSQQDTL
AFP29	300-308	TECCKLTTL
AFP30	37-46	CTAEISLADL
AFP31	209-218	VTKELRESSL
AFP32	284-293	IMSYICSQQD
AFP33	232-240	TRTFQAITV
AFP34	419-427	FQKLGEYYL
AFP35	372-380	RVAKGYQEL
AFP36	34-43	SYQCTAEISL
AFP37	549-557	KQEFLINLV
AFP38	1-9	MKWVESIFL
AFP39	492-500	PVNPGVGQC
AFP40	476-484	AADIIIGHL

TABLE I

HUMAN AFP PEPTIDE SEQUENCES

Page 2

Peptide Designation Name	Residues in hAFP Protein	Amino Acid Sequence
AFP41	140-148	QVPEPVTSC
AFP42	306-315	TTLERGQCI
AFP43	453-461	KMAATAATC
AFP44	539-548	QAQGVALQTM
AFP45	235-243	FQAITVTKL
AFP46	380-388	LLEKCFQTE
AFP47	433-441	VAYTKKAPQ
AFP48	403-411	KYIQESQAL
AFP49	542-550	GVALQTMKQ
AFP50	585-593	GQEQEVCFA
AFP51	117-126	SEEGRHNCFL
AFP52	169-178	RHPFLYAPTI
AFP53	253-262	TEIQKLVLDV
AFP54	360-369	RRHPQLAVSV
AFP55	423-432	GEYYLQNAFL
AFP56	507-516	NRRPCFSSLV
AFP57	545-554	LQTMKQEFLI
AFP58	572-581	IADFSGLLEK
AFP59	577-586	GLLEKCCQGQ
AFP60	294-302	TLSNKITEC
AFP61	278-287	LQDGEKIMSY
AFP62	417-425	GLFQKLGEY
AFP63	24-33	NEYGIASILD
AFP64	65-74	KMVKDALTAI
AFP65	350-358	FLASFVHEY
AFP66	52-60	AQFVQEATY

respect to the amino acid sequence of the hAFP molecule, SEQ ID NO:1

Next, each of the sixty-six peptides was tested for its ability to bind in a concentration dependent way to HLA A2.1 and, thereby, to stabilize HLA A2.1 in a T2 cell stabilization assay as follows. Each peptide was incubated overnight with TAP1 and TAP2 deleted T2 cells that had been incubated at room temperature the previous night to increase cell surface MHC class I molecule expression. Each peptide was tested for its ability to bind the HLA A2.1 molecule over a range of peptide concentrations, from 0.1 μM-100 μM. In the T2 cell line, only MHC molecules that are filled with eight to ten mer peptides are stable on the cell surface. Stability of HLA A2.1 was assayed by flow cytometry after staining the T2 cells with anti-HLA A2 antibody BB7.2 (ATCC) and goat antimouse-FITC. As positive controls for binding, the FLU matrix peptide (residues 58-66, GILGFVFTL, of FLU matrix 1 protein) and the MART-1 peptide (residues 27-35, AAGIGILTV, of MART-1, GenBank accession no. U06452 for the whole protein) were used. The FLU matrix peptide consistently stabilized the A2.1 molecules on T2 cells at concentrations of 0.5μM.

Referring now to Table II, there is shown a list of twenty-two of the sixty-six hAFP peptides. Column 1 lists the peptide designation number, column two identifies the residues of the hAFP sequence, SEQ ID NO:1, represented by the peptide sequence, and column three identifies the number of anchor residues within the sequence.

Column four of Table II lists the minimum concentration of peptide required to bind HLA 2.1 on T2 cells. As can be seen, six of the ten "strong" peptide sequences and seven of the forty-three "intermediate" peptide sequences showed binding ability to HLA 2.1. Further, none of the thirteen "weak" peptide sequences showed binding ability to HLA A2.1.

Further, each of the sixty-six peptides was also tested for their rate of dissociation from class I molecules over time in an EBV lymphoblastoid cell off-kinetics assay, because it has been found that the off-kinetics, that is the dissociation rate, of a peptide bound to a class I molecule is significantly predictive of the immunogenicity of that peptide. For example, for non-self binding peptides such as viral peptides HPV 16 E7, EBV LMP2, FLU M1 and HIV pol., it has been found that the strongest binding peptides showing the slowest off-kinetics were the most immunogenic. Further, it has been found that a number of known self-protein, immunogenic epitopes from melanoma antigens such as gp 100, MART-1, had one anchor reside and less stable binding affinity by a soluble class I reconstitution assay, but very slow off kinetics. See, for example, Bakker, A.B., et al.,

Analogues of CTL epitopes

TABLE II

HUMAN AFP PEPTIDE SEQUENCES & BINDING CHARACTERISTICS

Peptide Designation Name	Residues in hAFP Protein	Number of Anchor Residues	Minimum Concentration of Peptide to Stabilize A2 on T2 cells	Time of Peptide Stability on Lymphoblastoid Cells
AFP3	218-216	2	0.5μM	(n.s.) (not stable)
AFP4	257-265	2	0.5μM	(n.s.)
AFP5	158-166	2	0.1μ	>6 hrs
AFP6	135-143	1	(n.s.) (not stable)	>6 hrs
AFP7	12-20	1	50 μM	>6 hrs
AFP8	54-62	1	(n.s.)	>2 hrs
AFP13	404-412	1	10.0μM	>2 hrs
AFP14	441-450 (10 mer)	2	50.0μM	>2 hrs
AFP16	211-219	1	(n.s.)	2 hrs
AFP17	514-522	2	1.0μM	(n.s.)
AFP18	178-186	1	(n.s.)	>6 hrs
AFP22	547-56 (10 mer)	2	(n.s.)	4 hrs
AFP23	555-563	1	(n.s.)	>6 hrs
AFP28	287-295	1	50μM	2 hrs
AFP34	419-427	1	100μM	(n.s.)
AFP37	549-557	1	(n.s.)	<2 hrs
AFP38	1-9	1	0.5μM	<2 hrs
AFP39	492-500	0	(n.s.)	>6 hrs
AFP45	235-243	1	100μM	>6 hrs
AFP49	542-550	1	(n.s.)	>6 hrs
AFP60	294-302	1	50μM	(n.s.)
AFP64	65-74 (10 mer)	2	100μM	(n.s.)
CONTROLS:				
MART-1 peptide	Residues 27-35 of MART-1	1	50μM	2 hrs
FLU matrix peptide	Residues 58-66 of FLU matrix 1 protein	2	0.5μM	>6 hrs

with improved MHC class-I binding capacity elicit anti-melanoma CTL recognizing the wild-type epitope. Int J Cancer, 1997. 70(3): p. 302-9; and van der Burg, S.H., et al., *Do epitopes derived from autoantigens display low affinity for MHC class I? (letter)*. Immunol Today, 1997. 1892): p. 97-98; each incorporated herein by reference in their entirety.

The EBV lymphoblastoid cell off-kinetics assay was performed as disclosed in van der Burg, S.H., et al., *Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability*. J. Immunology, 1996. 156(9): p.3308-3314, incorporated herein by reference in its entirety. Briefly, HLA A2.1 EBV lymphoblastoid cells were stripped of surface class I peptides and β2 microglobulin in a mild pH 3.2 acid buffer which renders MHC molecules unstable. Each peptide was immediately pulsed onto the stripped cells in excess at 200 μM for 1 hour in the presence of β2 microglobulin. Excess unbound peptide was then washed off and the cells were incubated at 37°C and followed for 0, 2, 4 and 6 hours. The cells were washed at the end of each time point and stained for HLA A2 with the BB7.2 antibody. The peptide-class I complex was considered stable if the mean fluorescence intensity increased at least 1.5-fold from cells that were stripped but not pulsed with peptide.

Both the T2 cell stabilization assay and the EBV lymphoblastoid cell off-kinetics assay were performed for each peptide at least twice. Referring again to Table II, column 5 shows the time of peptide stability on the EBV lymphoblastoid cells. As can be seen, only three of the strong peptides (AFP5, AFP14 and AFP22), twelve of the forty-three intermediate peptides (including AFP49) and one of the weak peptides showed a level of slow off-kinetics. Taking into consideration both the T2 cell stabilization assay and the EBV lymphoblastoid cell off-kinetics assay, it can be seen that seven of the peptide sequences that gave the best results in both assays were AFP5, AFP7, AFP13, AFP14, AFP28, AFP38 and AFP45.

Next, the peptides listed in column 1 of Table III were then used to generate peptide specific CTL *in vitro* by the method disclosed in Plebanski, M. et al., *Induction of peptide-specific primary cytotoxic T lymphocyte responses from human peripheral blood*. Eur J. Immunol. 1995. 25(6): p. 1783-7 and the CTL were tested for their ability to lyse A2.1-positive, AFP-positive hepatocellular carcinoma cells. Lysis would suggest that the peptide is a naturally processed, immunogenic epitope of human AFP and potentially a target antigen. HLA A2.1 donors and cell lines were screened with the BB7.2 (HLA A2) antibody

(ATCC) and confirmed and subtyped by PCR and direct sequence analysis by the UCLA Tissue Typing

TABLE III

HUMAN AFP PEPTIDE CYTOTOXICITY

Peptide Designation Number	CTL Culture CD4/CD8 Phenotype	T2+Specific Peptide Cytotoxicity		AFP+/HLA A2.1 + HepG2 Cytotoxicity	
AFP3	CD8+	50:1 10:1	74% 28%	20:1 5:1	7% 4%
AFP4	CD8+	50:1 10:1	94% 15%	20:1 5:1	7% 4%
AFP5	CD8+	50:1 10:1	75% 41%	20:1 5:1	7% 4%
AFP6	CD8+	50:1 10:1	52% 17%	(n.t.)	
AFP7	CD8+	50:1 10:1	22% 0	50:1 10:1	15% 6%
AFP22	CD8+/CD4+	20:1 5:1	56% 13%	20:1 5:1	24% 9%
AFP23	CD8+/CD4+	20:1 5:1	45% 10%	20:1 5:1	15% 2%
AFP37	CD8+/CD4+	25:1 5:1	32% 16%	25:1 5:1	2% 0
AFP38	CD8+/CD4+	50:1 10:1	54% 18%	50:1 10:1	12% 0
AFP39	CD8+/CD4+	50:1 10:1	54% 18%	50:1 10:1	29% 12%
AFP45	CD8+/CD4+	50:1 10:1	25% 14%	50:1 10:1	27% 9%
AFP49	CD8+/CD4+	50:1 10:1	46% 23%	50:1 10:1	45% 17%
Residues 58-66 of FLU	CD8+	50:1 10:1	100% 45%	n.t.	
Residues 27-35 of MART-1	CD8+	50:1 10:1	100% 80%	n.t.	

Laboratory, according to techniques known to those with skill in the art. Briefly, peptide specific CTL were generated to the peptides listed in Table III AFP peptides as follows. $2x10^7$ peripheral blood mononuclear cells (PBMC) from a normal A2.1 donor were purified by Ficoll gradient. These PBMC were pulsed with 50μg/ml peptide in 1 ml serum-free media for 90 minutes at 37°C. The cells were then rinsed once and placed in a 24-well plate at 3 x 10^6 PBMC in 1.5 ml of 10% autologous serum RPMI medium per well on day 0 with IL-7 (10 ng/ml) and KLH (4.5 μg/ml) in RPMI/10% autologous serum. CTL were restimulated weekly by removing the non-adherent cells and adding them to fresh, peptide-pulsed, washed and irradiated PBMC at 1:1 PBMC to CTL ratio. IL-2 was added twice weekly at 10 units/ml.

After three weeks of culture, the putative hAFP peptide-generated CTL were tested for cytotoxicity in a standard 4 hour ^{51}Cr-release assay. The CTL were tested for peptide-specific killing against T2 cells pulsed with the specific hAFP peptide used to generate the CTL and compared with T2 cells pulsed with the FLU matrix peptide or the MART-1 peptide as controls. Non-specific NK killing was assessed with the NK sensitive target K562. The CTL were also tested against the HLA A2.1-positive, AFP-positive human hepatocellular carcinoma cell line, HepG2.

Referring now to Table III, there are shown the cytotoxicity results of tests for the twelve AFP peptide sequences used to generate CTL from normal donors that gave positive peptide cytotoxicity results. Column 2 shows the CD4/CD8 phenotype of the bulk lymphocyte culture. Columns 3 and 4 show, respectively, the levels of cytotoxicity against peptide-pulsed T2 cells and HepG2 targets with their effector (CTL) to target ratio (E:T).

As can be seen in Table III, peptides AFP22, AFP39, AFP45 and AFP49 demonstrated high levels of specific killing of AFP+, HLA A2.1+ HepG2 cells. It can be noted that AFP22 and AFP49 have a four amino acid overlap, residues 547-550 of hAFP SEQ ID NO:1. Further, AFP22 has a two amino acid overlap, residues 555-556 of SEQ ID NO:1, with AFP23, which showed marginal HepG2 killing.

CTL generated using AFP49 were retested and the HepG2 cytotoxicity was maintained. Further, new AFP49 peptide-generated CTL cultures were made using two different, normal HLA A2.1 donors. Additional targets were used to confirm that the cytotoxicity observed using AFP49 was AFP antigen-specific and class I restricted.

Referring now to Figure 1, there is shown representative data of these tests.

First, to confirm that the observed cytotoxicity was class I restricted, anti-β2 microglobulin antibody was used to block the CTL-T cell receptor interaction on HepG2 cells. This resulted in a significant reduction in HepG2 lysis. Next, to eliminate non-specific NK/LAK killing, a 40-fold excess of unlabeled (cold) K562 cells was added. This did not result is a significant reduction in HepG2 lysis. Further, MHC class I expression was upregulated on HepG2 cells by overnight incubation with γIFN (50 units/ml). As can be seen, MHC class I expression upregulation increased HepG2 lysis. Also, an AFP+, HLA A2.1-negative hepatocellular carcinoma cell line, Hep3B, that is, a class I-mismatched hepatocellular carcinoma cell line, was also used as a target. AFP49 CTL lysed these Hep3B targets at a very low level. This small amount of observed Hep3B lysis was eliminated by adding an excess of cold K562 cells, in contrast to the retention of specific killing of HepG2 when cold K562 cells were added.

Referring now to Figure 2, there is shown a bar graph of percent specific lysis versus targets for a standard chromium release assay of CTL generated from peptide-pulsed PBMC from a normal, HLA A2.1 donor assayed against both peptide targets and AFP targets. To confirm the peptide specificity of the CTL, each culture was tested against T2 cells pulsed with the specific peptide from which the CTL culture was made, left most bars, and compared with T2 cells pulsed with a different HLA A2.1 binding peptide as a control, second group of bars from the left. As can be seen, the AFP49 peptide culture, the AFP49V9 peptide culture, the AFP5 peptide culture and the control FLU matrix peptide culture all showed peptide specificity by lysis of T2 cells pulsed with the specific peptide, but not against T2 cells pulsed with a different peptide.

Referring again to Figure 2, each of these peptide-specific CTL cultures was also tested for killing of M202 (HLA A2.1+/AFP-) melanoma cells transduced with either AdVhAFP or the control AdVRR5. Each peptide-specific CTL culture of the AFP peptides AFP5 and AFP49, as well as the peptides AFP49L9, SEQ ID NO:2 (GVALQTMKL) and AFP49V9, SEQ ID NO:3 (GVALQTMKV), which have a single amino acid substitution in position 9 of AFP49, show significantly more killing of M202 cells transduced with AdVhAFP than killing of M202 cells transduced with the control RR5. The FLU peptide-specific CTL cultures killed both M202/AdVhAFP and M202/RR5 with similar, background levels of cytotoxicity.

M202 cells are known to correctly process and present the HLA A2.1-

restricted, immunodominant MART-1 peptide. Therefore, they are an ideal cell line to transduce with AdVhAFP and expect that the correct HLA A2.1-restricted epitopes from AFP will be processed and presented on the surface. Hence, this experiment demonstrates that AFP5, AFP49, AFP49L9, SEQ ID NO:2 and AFP49V9, SEQ ID NO:3 are naturally processed and presented peptides that can be used to target CTL to kill AFP+ tumors. Further, as can be seen AFP49L9, SEQ ID NO:2, and AFP49V9, SEQ ID NO:3, peptide-specific CTL cultures kill M202/AdVhAFP even more effectively than AFP49 peptide-specific CTL cultures and, therefore, AFP49L9, SEQ ID NO:2, and AFP49V9, SEQ ID NO:3, are improved peptides for targeting the immune response to AFP+ cells.

Thus, as can be appreciated from this disclosure, the present invention includes preventing or treating a cancer in a mammal, including a human, where the cancer cells bear at least part of the alphafetoprotein molecule as a surface marker. The prevention or treatment is accomplished by administering to the mammal a composition including a peptide that comprises at least part of the alphafetoprotein molecule or a peptide that has been produced by substitution of or other alterations to at least part of the alphafetoprotein molecule. These peptides include AFP5, AFP7, AFP13, AFP14, AFP18, AFP22, AFP23, AFP28 AFP38, AFP39, AFP45, AFP49, AFP49L9, SEQ ID NO:2, and AFP49V9, SEQ ID NO:3.

2) IMMUNIZATION OF MAMMALS USING ALPHAFETOPROTEIN cDNA TO CREATE AN IMMUNE RESPONSE TO CELLS BEARING ALPHAFETOPROTEIN ON THEIR SURFACE, INCLUDING HEPATOCELLULAR CANCER CELLS

Immunizing mammals with alphafetoprotein cDNA creates an immune response that is partially or fully protective against challenges with tumor cells bearing alphafetoprotein on their surface, including hepatocellular cancer cells. This effect was demonstrated as follows:

Human alphafetoprotein cDNA was produced as follows. First, human alphafetoprotein cDNA was generated by PCR techniques from total RNA made from Hep3B cells (available from ATCC) by the Trizol method (Life Technologies, Gaithersburg, MD) according to the manufacture's instructions) and by the RNAzolB method (TelTest, Friendswood, TX). Approximately 1 μg of total RNA was used in an RT-PCR reaction using the Perkin Elmer RT-PCR kit and AFP-specific primers based on the published

sequence. The 5' primer was 5' GCA ACC ATG AAG TGG GT. The 3' primer was 5' AAC TCC CAA AGC AGC ACG AGT. The primers included the entire coding region (ATG to the stop codon) with a restriction endonuclease site XbaI incorporated into the primer, and ending with six bases (CTC TCT) to facilitate enzyme cleavage after PCR. Primer sequences were synthesized by Operon Technologies, 50 nM scale, unpurified.

The human alphafetoprotein PCR cDNA products produced above were analyzed on an agarose gel to check their size. Correctly sized products were purified on a Qiagen PCR-quick clean-up column, digested with the XbaI enzyme whose site was designed into the primers, and used in a cloning reaction into either pRcCMV (for human) or pCR3.1 (for murine) mammalian expression vectors (Invitrogen, Carlsbad, CA) according to techniques known to those with skill in the art. Positive plasmids were identified by miniprep analysis. These positive plasmids were maxiprepped and an aliquot was sequenced by the DNA sequencing core facility at UCLA to confirm the sequence identity of the inserts. The sequence data was for one strand only, and confirmed the identity of the AFP inserts. Therefore, the human AFP cDNA cloned was identical to the human AFP published sequence, GenBank accession Nos J00077, J00076, V01514, bases 48-1877, SEQ ID NO:1.

Murine AFP cDNA (mAFP cDNA) was cloned using corresponding methods to the methods disclosed above used to clone human AFP cDNA, but with mouse-specific primers. The 5' murine specific primers was 5' GCC ATG AAG TGG ATC ACA. The 3' murine specific primer was TTA AAC GCC CAA AGC ATC A. The mouse AFP-positive cell line used to isolate total RNA was Hepa16. All stable transfectants and intramuscular injection experiments disclosed herein were performed with cDNA clones containing the signal-sequence. The mouse sequence is bases 42-1859 of Embl V00743, SEQ ID NO:4.

Next, the mAFP cDNA was placed in the eucaryotic expression vector VR1012 (Vical, Inc., San Diego, CA). The VR1012 expression vector contains the strong constitutive CMV immediate early promoter/enhancer, including an intron for enhanced expression, a BGH termination and poly A sequences for *in vivo* expression.

C57BL/6 mice were given im injections of 100μg VR1012 containing the mAFP cDNA or saline as a control once a week for three weeks. One week after the last injection, both the VR1012 mAFP cDNA immunized mice and the unimmunized group of control mice were challenged with $4x10^6$ viable BWIC3 hepatocellular carcinoma cells obtained from a single cell suspension of progressively growing tumors in syngeneic mice.

BWIC3 is a mAFP-positive murine cell line.

Referring now to Figure 3, it can be seen that immunized animals (open boxes), showed a delayed tumor growth or complete protection compared with control animals (closed squares). These finding were replicated several times. In a corresponding experiment, im injections of a plasmid vector expressing the MART-1 melanoma antigen did not protect animals from a BWIC3 hepatocellular carcinoma cell challenge (data not shown).

In another group of experiments, a surrogate murine hepatocellular carcinoma line was constructed by stably transfecting the EL4 (H-2^b) lymphoma with mAFP cDNA. The tumor line EL4(mAFP) has the same *in vivo* growth kinetics as the parental EL4 cell line. Using RT-PCR, it appears that the EL4(mAFP) tumor cell line produces 1% or less of the levels of AFP as BWIC3 hepatocellular carcinoma cell line.

C57BL/6 mice were given im injections of 100μg VR1012 containing the mAFP cDNA or saline as a control once a week for three weeks. One week after the last injection, both the VR1012 mAFP cDNA immunized mice and the unimmunized group of control mice were challenged with 7.5×10^5 viable EL4(parental) or EL4(mAFP) cells.

Referring now to Figure 4, it can be seen that immunized animals (open boxes), and control animals (closed circles) showed no difference when challenged with EL4(parental) cells ($p=0.07$, student's T test). However, as can be seen in Figure 5, immunized animals (open boxes), did show partial protection compared with control animals (closed circles), when challenged with EL4(mAFP) cells ($p=0.07$, student's T test). These findings were also replicated several times.

In an additional series of experiments, protection against challenges with cells bearing alphafetoprotein on their surface was demonstrated using stably-transfected mouse fibrosarcoma cell lines as a surrogate. First, stably-transfected mouse fibrosarcoma cell lines were produced by either the DOTAP lipofection method (Boehringer Mannheim) according to the manufacture's instruction) and a $CaPO_4$ precipitation method (according to techniques well known to those with skill in the art). In summary, the DOTAP lipofection method used 1×10^5 cells per well in a 6-well plate, adhered overnight the previous night. 2.5 ug plasmid (murine AFP pCR3.1) was mixed in 25 μl of 20 mM Hepes and 15 μl lipid in 50 μl Hepes at room temperature for 15 min. This was diluted into 1 ml of culture medium (RPMI1640 containing 10% fetal calf serum and antibiotics), and added to the cells in the wells. After 4-6 hours, the solution was replaced with 2 ml fresh culture medium. After 48-72 hours,

selection was started with G418 (geneticin) @ 500 μg/ml (total concentration, 75% active). After 2-3 weeks of selection, any potential transfectants were tested by RT-PCR for expression of mouse AFP RNA, neo-RNA and semi-quantified with murine APRT gene expression.

The effectiveness of AFP immunization in preventing tumor production in mammals was demonstrated as follows. Mouse AFP-pCR3.1 plasmid and mouse AFP-AdVShuttle vector plasmid (pLpA CMV) were prepared according to techniques known to those with skill in the art, and mouse AFP-Vical vector VR1012 was constructed. Murine fibrosarcoma cell lines FSA, NFSA, MCAK and SVEC were stably transfected with mAFP PCR3.1, as above.

C3H mice were immunized by weekly intramuscular injections for three weeks of plasmid DNA using mouse AFP-AdVShuttle vector neo-containing expression plasmids prepared endotoxin-free with a Qiagen plasmid prep kit (50 μg plasmid in 50 μl PBS). The C3H mice were then challenged with FSA C3H background fibrosarcoma cells stably transfected with AFP-expressing vector or FSA C3H background fibrosarcoma cells stably transfected with the neo-expressing vector only, to determine whether an AFP anti-self antigen response could be generated or whether use of stable transfectants expressing neomycin would create an anti-neo (non-self antigen) response that would mask the AFP response. Tumor cells were passaged in vivo, and a single cell suspension was used for tumor challenges.

Referring now to Figure 6, it can be seen that by day 18 post-tumor challenge, only one of the immunized CH3 mice (lower closed squares) challenged with FSA C3H background fibrosarcoma cells stably transfected with AFP-expressing vector showed any tumor growth (a 3 mm x 3 mm tumor), while the remaining four immunized CH3 mice (closed circles) challenged with FSA C3H background fibrosarcoma cells stably transfected with AFP-expressing vector did not show any sign of tumor growth. By contrast, two of the five unimmunized C3H mice (upper closed squares) challenged with FSA C3H background fibrosarcoma cells stably transfected with AFP-expressing vector showed any tumor growth (mean 6.8 mm^2). FSA parental tumor cells and neo-vector-FSA cells grew similarly in both immunized (closed diamonds) and unimmunized (closed inverted triangles) CH3 mice. This protocol was repeated and similar results were obtained. (Data not shown.)

A second experiment was performed using C57L/J ("leaden") mice from

Jackson Labs (Bar Harbor Maine). These mice were immunized with a plasmid vector from Vical (VR1012) that does not contain neomycin and, therefore, synthesized only the mouse AFP gene. The C57L/J mice were challenged with a murine syngeneic tumor cell line, BWIC3 from ATCC. These BWIC3 cells synthesize a much higher level of mouse AFP than the stably-transfected murine fibrosarcoma cells produced as disclosed above.

The C57L/J mice were immunized as described above using mAFP-Vical vector and a tumor challenge of 1 x 10^6 BWIC3 cells per mouse was made subcutaneously. Referring now to Figure 7, it can be seen that at day 14 post-tumor challenge, unimmunized C57L/J mice (closed squares) had tumors that averaged two times larger than the tumors in immunized C57L/J mice (closed circles).

In a third experiment, additional C57L/J mice were immunized with a plasmid vector (VR1012) that synthesized only the mouse AFP gene and challenged with 1 x 10^6 BWIC3 cells as disclosed above. Referring now to Figure 8, it can be seen that by seventeen days post challenge, all five unimmunized (closed circles and closed squares) mice had tumors, averaging 11.4 mm^2 in diameter. By contrast, three of the five mice immunized (closed triangles) with mAFP-Vical had tumors averaging 9 mm^2, one immunized mouse (closed diamonds) had a small 3 mm^2 tumor, and one mouse (closed inverted triangles) did not show any tumor.

Therefore, as can be appreciated from this disclosure, the present invention includes preventing or treating a cancer in a mammal, including a human, where the cancer cells bear at least part of the alphafetoprotein molecule as a surface marker. The prevention or treatment is accomplished by administering to the mammal a composition including at least a portion of the alphafetoprotein cDNA to create an immune response against at least part of the alphafetoprotein molecule.

3) IMMUNIZATION OF MAMMALS USING GENETICALLY-ENGINEERED DENDRITIC CELLS TO CELLS BEARING ALPHAFETOPROTEIN ON THEIR SURFACE, INCLUDING HEPATOCELLULAR CANCER CELLS

Immunizing mammals with dendritic cells transduced with a recombinant adenovirus vector that expresses murine AFP (AdVmAFP) alphafetoprotein cDNA creates an immune response that is partially or fully protective against challenges with hepatocellular cancer cells. This effect was demonstrated as follows:

First, a recombinant adenovirus vector that expresses murine AFP

(AdVmAFP) was constructed, according to techniques known to those with skill in the art. See for example, Ribas, A., L. H. Butterfield, W. H. McBride, S. M. Jilani, L. A. Bui, C. M. Vollmer, R. Lau, V. B. Dissette, B. Hu, A. Y. Chen, J. A. Glaspy, and J. S. Economou. 1997. Genetic immunization for the melanoma antigen MART-1/Melan-A using recombinant adenovirus-transduced murine dendritic cells. *Cancer Res 57:2865*; and Toloza, E. M., K. Hunt, S. Swisher, W. McBride, R. Lau, S. Pang, K. Rhoades, T. Drake, A. Belldegrun, J. Glaspy, and J. S. Economou. 1996. In vivo cancer gene therapy with a recombinant interleukin-2 adenovirus vector. *Cancer Gene Ther 3:11*, incorporated herein by reference in its entirety. Then, dendritic cells were generated from C57BL/6 bone marrow differentiated for seven days in GM-CSF and IL-4, according to techniques known to those with skill in the art. See for example, Ribas, A., L. H. Butterfield, W. H. McBride, S. M. Jilani, L. A. Bui, C. M. Vollmer, R. Lau, V. B. Dissette, B. Hu, A. Y. Chen, J. A. Glaspy, and J. S. Economou. 1997. Genetic immunization for the melanoma antigen MART-1/Melan-A using recombinant adenovirus-transduced murine dendritic cells. *Cancer Res 57:2865*; and Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med 176:1693*, incorporated herein by reference in their entirety.

Referring now to Figure 9, there is shown an RT-PCR analysis of mRNA isolated from murine DC transduced with AdVmAFP at various multiplicities of infection (MOI). Reading from left to right, lane 1 shows gel size standards; lane 2 shows the results for mAFP negative cells used as a negative control; lane 3 shows the results for murine dendritic cells used as a negative control; lanes 4-7 show the results for murine dendritic cells transduced with AdVmAFP at a MOI of 10, 100, 1,000 and 5,000, respectively; lane 8 shows the results for BWIC3 cells used as a positive control (upper most line at approximately 1.9 kb); and lane 9 shows the results for double distilled water (DDW), as a no-template control for PCR contamination. As can be seen, the recombinant adenovirus vector that expresses murine AFP (AdVmAFP) successfully transduced the dendritic cells.

Next, three groups of five C57BL/6 mice were prepared by giving one iv injection per week for two weeks of either $5x10^5$ dendritic cells transduced at an MOI of 100 with AdVmAFP, RR5 (an empty E1-deleted adenovirus), or untreated dendritic cells. These three groups of mice and one group of uninjected mice serving as a control were challenged

with $7.5x10^5$ EL4(AFP) one week after the last injection. The results are shown in Figure 10. As can be seen, neither the mice injected with RRS (closed upright triangles), or untreated dendritic cells (closed inverted triangles), nor the control mice (closed circles), showed protection against the tumor challenge. By contrast, mice injected with $5x10^5$ dendritic cells transduced at an MOI of 100 with AdVmAFP (closed squares), showed partial protection against the tumor challenge.

Further, another group of five mice was prepared by giving one iv injection per week for two weeks of $5x10^5$ dendritic cells transduced at an MOI of 100 with AdVmAFP. The response of this group to challenge with $4x10^6$ BWIC3 tumor cells one week after the last injection of transduced dendritic cells was compared to the response of a group of similar but uninjected control mice. The results of this test are shown in Figure 11. As can be seen, the immunized mice (closed squares) showed significant protection against the tumor challenge compared to the control mice (closed circles), demonstrating the effectiveness of the treatment with transduced dendritic cells.

Therefore, as can be appreciated from this disclosure, the present invention includes preventing or treating a cancer in a mammal, including a human, where the cancer cells bear at least part of the alphafetoprotein molecule as a surface marker. The prevention or treatment is accomplished by administering to the mammal a composition including immune system cells, such as dendritic cells, transduced with a recombinant vector that expresses alphafetoprotein cDNA.

EXAMPLE I

TREATMENT OF HEPATOCELLULAR CARCINOMA IN A MAMMAL

According to one embodiment of the present invention, there is provided a method for treating hepatocellular carcinoma in a human by creating an immune response in the human to at least part of the alphafetoprotein molecule. The method includes immunizing the human in a method similar to one of the methods disclosed herein or a corresponding method, or genetically manipulating the human to produce an immune response to alphafetoprotein. In a preferred embodiment, the human with hepatocellular carcinoma is immunized to produce an immune response to at least part of the human alphafetoprotein molecule, such as to AFP5, AFP7, AFP13, AFP14, AFP18, AFP22, AFP23, AFP28 AFP38, AFP39, AFP45 or AFP49. This immunization causes the human's immune system to attack the hepatocellular carcinoma cells having that portion of the

alphafetoprotein molecule on their surface.

Although the present invention has been discussed in considerable detail with reference to certain preferred embodiments, other embodiments are possible. Therefore, the spirit and scope of the appended claims should not be limited to the description of preferred embodiments contained herein.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: PREVENTION AND TREATMENT OF HEPATOCELLULAR CANCER
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(B) COMPUTER: IBM compatible
(C) OPERATING SYSTEM: Windows 95
(D) SOFTWARE: WordPerfect for Windows version 8.0
(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: filed herewith
(B) FILING DATE: 13 February, 1998
(C) CLASSIFICATION: to be assigned
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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2032 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double stranded
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(ix) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TCCATATTGT GCTTCCACCA CTGCCAATAA CAAAATAACT AGCAACC ATG AAG TGG        56
                                                    Met Lys Trp
                                                      1

GTG GAA TCA ATT TTT TTA ATT TTC CTA CTA AAT TTT ACT GAA TCC AGA       104
Val Glu Ser Ile Phe Leu Ile Phe Leu Leu Asn Phe Thr Glu Ser Arg
  5                  10                  15

ACA CTG CAT AGA AAT GAA TAT GGA ATA GCT TCC ATA TTG GAT TCT TAC       152
Thr Leu His Arg Asn Glu Tyr Gly Ile Ala Ser Ile Leu Asp Ser Tyr
 20                  25                  30                  35

CAA TGT ACT GCA GAG ATA AGT TTA GCT GAC CTG GCT ACC ATA TTT TTT       200
Gln Cys Thr Ala Glu Ile Ser Leu Ala Asp Leu Ala Thr Ile Phe Phe
                 40                  45                  50

GCC CAG TTT GTT CAA GAA GCC ACT TAC AAG GAA GTA AGC AAA ATG GTG       248
Ala Gln Phe Val Gln Glu Ala Thr Tyr Lys Glu Val Ser Lys Met Val
             55                  60                  65

AAA GAT GCA TTG ACT GCA ATT GAG AAA CCC ACT GGA GAT GAA CAG TCT       296
Lys Asp Ala Leu Thr Ala Ile Glu Lys Pro Thr Gly Asp Glu Gln Ser
         70                  75                  80
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25

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TCA GGG TGT TTA GAA AAC CAG CTA CCT GCC TTT CTG GAA GAA CTT TGC          344
Ser Gly Cys Leu Glu Asn Gln Leu Pro Ala Phe Leu Glu Glu Leu Cys
 85                  90                  95

CAT GAG AAA GAA ATT TTG GAG AAG TAC GGA CAT TCA GAC TGC TGC AGC          392
His Glu Lys Glu Ile Leu Glu Lys Tyr Gly His Ser Asp Cys Cys Ser
100                 105                 110                 115

CAA AGT GAA GAG GGA AGA CAT AAC TGT TTT CTT GCA CAC AAA AAG CCC          440
Gln Ser Glu Glu Gly Arg His Asn Cys Phe Leu Ala His Lys Lys Pro
                120                 125                 130

ACT CCA GCA TCG ATC CCA CTT TTC CAA GTT CCA GAA CCT GTC ACA AGC          488
Thr Pro Ala Ser Ile Pro Leu Phe Gln Val Pro Glu Pro Val Thr Ser
            135                 140                 145

TGT GAA GCA TAT GAA GAA GAC AGG GAG ACA TTC ATG AAC AAA TTC ATT          536
Cys Glu Ala Tyr Glu Glu Asp Arg Glu Thr Phe Met Asn Lys Phe Ile
        150                 155                 160

TAT GAG ATA GCA AGA AGG CAT CCC TTC CTG TAT GCA CCT ACA ATT CTT          584
Tyr Glu Ile Ala Arg Arg His Pro Phe Leu Tyr Ala Pro Thr Ile Leu
    165                 170                 175

CTT TGG GCT GCT CGC TAT GAC AAA ATA ATT CCA TCT TGC TGC AAA GCT          632
Leu Trp Ala Ala Arg Tyr Asp Lys Ile Ile Pro Ser Cys Cys Lys Ala
180                 185                 190                 195

GAA AAT GCA GTT GAA TGC TTC CAA ACA AAG GCA GCA ACA GTT ACA AAA          680
Glu Asn Ala Val Glu Cys Phe Gln Thr Lys Ala Ala Thr Val Thr Lys
                200                 205                 210

GAA TTA AGA GAA AGC AGC TTG TTA AAT CAA CAT GCA TGT GCA GTA ATG          728
Glu Leu Arg Glu Ser Ser Leu Leu Asn Gln His Ala Cys Ala Val Met
            215                 220                 225

AAA AAT TTT GGG ACC CGA ACT TTC CAA GCC ATA ACT GTT ACT AAA CTG          776
Lys Asn Phe Gly Thr Arg Thr Phe Gln Ala Ile Thr Val Thr Lys Leu
        230                 235                 240

AGT CAG AAG TTT ACC AAA GTT AAT TTT ACT GAA ATC CAG AAA CTA GTC          824
Ser Gln Lys Phe Thr Lys Val Asn Phe Thr Glu Ile Gln Lys Leu Val
    245                 250                 255

CTG GAT GTG GCC CAT GTA CAT GAG CAC TGT TGC AGA GGA GAT GTG CTG          872
Leu Asp Val Ala His Val His Glu His Cys Cys Arg Gly Asp Val Leu
260                 265                 270                 275

GAT TGT CTG CAG GAT GGG GAA AAA ATC ATG TCC TAC ATA TGT TCT CAA          920
Asp Cys Leu Gln Asp Gly Glu Lys Ile Met Ser Tyr Ile Cys Ser Gln
                280                 285                 290

CAA GAC ACT CTG TCA AAC AAA ATA ACA GAA TGC TGC AAA CTG ACC ACG          968
Gln Asp Thr Leu Ser Asn Lys Ile Thr Glu Cys Cys Lys Leu Thr Thr
            295                 300                 305

CTG GAA CGT GGT CAA TGT ATA ATT CAT GCA GAA AAT GAT GAA AAA CCT         1016
Leu Glu Arg Gly Gln Cys Ile Ile His Ala Glu Asn Asp Glu Lys Pro
        310                 315                 320

GAA GGT CTA TCT CCA AAT CTA AAC AGG TTT TTA GGA GAT AGA GAT TTT         1064
Glu Gly Leu Ser Pro Asn Leu Asn Arg Phe Leu Gly Asp Arg Asp Phe
    325                 330                 335
```

```
AAC CAA TTT TCT TCA GGG GAA AAA AAT ATC TTC TTG GCA AGT TTT GTT      1112
Asn Gln Phe Ser Ser Gly Glu Lys Asn Ile Phe Leu Ala Ser Phe Val
340                 345                 350                 355

CAT GAA TAT TCA AGA AGA CAT CCT CAG CTT GCT GTC TCA GTA ATT CTA      1160
His Glu Tyr Ser Arg Arg His Pro Gln Leu Ala Val Ser Val Ile Leu
                360                 365                 370

AGA GTT GCT AAA GGA TAC CAG GAG TTA TTG GAG AAG TGT TTC CAG ACT      1208
Arg Val Ala Lys Gly Tyr Gln Glu Leu Leu Glu Lys Cys Phe Gln Thr
            375                 380                 385

GAA AAC CCT CTT GAA TGC CAA GAT AAA GGA GAA GAA GAA TTA CAG AAA      1256
Glu Asn Pro Leu Glu Cys Gln Asp Lys Gly Glu Glu Glu Leu Gln Lys
        390                 395                 400

TAC ATC CAG GAG AGC CAA GCA TTG GCA AAG CGA AGC TGC GGC CTC TTC      1304
Tyr Ile Gln Glu Ser Gln Ala Leu Ala Lys Arg Ser Cys Gly Leu Phe
    405                 410                 415

CAG AAA CTA GGA GAA TAT TAC TTA CAA AAT GCG TTT CTC GTT GCT TAC      1352
Gln Lys Leu Gly Glu Tyr Tyr Leu Gln Asn Ala Phe Leu Val Ala Tyr
420                 425                 430                 435

ACA AAG AAA GCC CCC CAG CTG ACC TCG TCG GAG CTG ATG GCC ATC ACC      1400
Thr Lys Lys Ala Pro Gln Leu Thr Ser Ser Glu Leu Met Ala Ile Thr
                440                 445                 450

AGA AAA ATG GCA GCC ACA GCA GCC ACT TGT TGC CAA CTC AGT GAG GAC      1448
Arg Lys Met Ala Ala Thr Ala Ala Thr Cys Cys Gln Leu Ser Glu Asp
            455                 460                 465

AAA CTA TTG GCC TGT GGC GAG GGA GCG GCT GAC ATT ATT ATC GGA CAC      1496
Lys Leu Leu Ala Cys Gly Glu Gly Ala Ala Asp Ile Ile Ile Gly His
        470                 475                 480

TTA TGT ATC AGA CAT GAA ATG ACT CCA GTA AAC CCT GGT GTT GGC CAG      1544
Leu Cys Ile Arg His Glu Met Thr Pro Val Asn Pro Gly Val Gly Gln
    485                 490                 495


TGC TGC ACT TCT TCA TAT GCC AAC AGG AGG CCA TGC TTC AGC AGC TTG      1592
Cys Cys Thr Ser Ser Tyr Ala Asn Arg Arg Pro Cys Phe Ser Ser Leu
500                 505                 510                 515

GTG GTG GAT GAA ACA TAT GTC CCT CCT GCA TTC TCT GAT GAC AAG TTC      1640
Val Val Asp Glu Thr Tyr Val Pro Pro Ala Phe Ser Asp Asp Lys Phe
                520                 525                 530

ATT TTC CAT AAG GAT CTG TGC CAA GCT CAG GGT GTA GCG CTG CAA ACG      1688
Ile Phe His Lys Asp Leu Cys Gln Ala Gln Gly Val Ala Leu Gln Thr
            535                 540                 545

ATG AAG CAA GAG TTT CTC ATT AAC CTT GTG AAG CAA AAG CCA CAA ATA      1736
Met Lys Gln Glu Phe Leu Ile Asn Leu Val Lys Gln Lys Pro Gln Ile
        550                 555                 560

ACA GAG GAA CAA CTT GAG GCT GTC ATT GCA GAT TTC TCA GGC CTG TTG      1784
Thr Glu Glu Gln Leu Glu Ala Val Ile Ala Asp Phe Ser Gly Leu Leu
    565                 570                 575

GAG AAA TGC TGC CAA GGC CAG GAA CAG GAA GTC TGC TTT GCT GAA GAG      1832
Glu Lys Cys Cys Gln Gly Gln Glu Gln Glu Val Cys Phe Ala Glu Glu
```

580 585 590 595

GGA CAA AAA CTG ATT TCA AAA ACT CGT GCT GCT TTG GGA GTT TAAATTA 1881
Gly Gln Lys Leu Ile Ser Lys Thr Arg Ala Ala Leu Gly Val
600 605

CTTCAGGGGA AGAGAAGACA AAACGAGTCT TTCATTCGGT GTGAACTTTT CTCTTTAATT 1941

TTAACTGATT TAACACTTTT TGTGAATTAA TGAAATGATA AAGACTTTTA TGTGAGATTT 2001

CCTTATCACA GAAATAAAAT ATCTCCAAAT G 2032

(2) INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acid residues
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(ix) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Val Ala Leu Gln Thr Met Lys Leu
1 5

(2) INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acid residues
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(ix) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gly Val Ala Leu Gln Thr Met Lys Val
1 5

(2) INFORMATION FOR SEQ ID NO:4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2009 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(ix) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCCCACTTCC AGCACTGCCT GCGGTGAAGG AACAAGCAGC CATGAAGTGG ATCACACCCG 60

CTTCCCTCAT CCTCCTGCTA CATTTCGCTG CGTCCAAAGC ATTGCACGAA AATGAGTTTG 120

GGATAGCTTC CACGTTAGAT TCCTCCCAGT GCGTGACGGA GAAGAATGTG CTTAGCATAG 180

CTACCATCAC CTTTACCCAG TTTGTTCCGG AAGCCACCGA GGAGGAAGTG AACAAAATGA 240

CTAGCGATGT GTTGGCTGCA ATGAAGAAAA ACTCTGGCGA TGGGTGTTTA GAAAGCCAGC 300

TATCTGTGTT TCTGGATGAA ATTTGCCATG AGACGGAACT CTCTAACAAG TATGGACTCT 360

CAGGCTGCTG CAGCCAAAGT GGAGTGGAAA GACATCAGTG TCTGCTGGCA CGCAAGAAGA 420

CTGCTCCGGC CTCTGTCCCA CCCTTCCAGT TTCCAGAACC TGCCGAGAGT TGCAAAGCAC 480

ATGAAGAAAA CAGGGCAGTG TTCATGAACA GGTTCATCTA TGAAGTGTCA AGGAGGAACC 540

CCTTCATGTA TGCCCCAGCC ATTCTGTCCT TGGCTGCTCA GTACGACAAG GTCGTTCTGG 600

CATGCTGCAA AGCTGACAAC AAGGAGGAGT GCTTCCAGAC AAAGAGAGCA TCCATTGCAA 660

AGGAATTAAG AGAAGGAAGC ATGTTAAATG AGCATGTATG TTCAGTGATA AGAAAATTTG 720

GATCCCGAAA CCTCCAGGCA ACAACCATTA TTAAGCTAAG TCAAAAGTTA ACTGAAGCAA 780

ATTTTACTGA GATTCAGAAG CTGGCCCTGG ATGTGGCTCA CATCCACGAG GAGTGTTGCC 840

AAGGAAACTC GCTGGAGTGT CTGCAGGATG GGGAAAAAGT CATGACATAT ATATGTTCTC 900

AACAAAATAT TCTGTCAAGC AAAATAGCAG AGTGCTGCAA ATTACCCATG ATCCAACTAG 960

GCTTCTGCAT AATTCACGCA GAGAATGGCG TCAAACCTGA AGGCTTATCT CTAAATCCAA 1020

GCCAGTTTTT GGGAGACAGA AATTTTGCCC AATTTTCTTC AGAGGAAAAA ATCATGTTCA 1080

TGGCAAGCTT TCTTCATGAA TACTCAAGAA CTCACCCCAA CCTTCCTGTC TCAGTCATTC 1140

TAAGAATTGC TAAAACGTAC CAGGAAATAT TGGAGAAGTG TTCCCAGTCT GGAAATCTAC 1200

CTGGATGTCA GGACAATCTG GAAGAAGAAT TGCAGAAACA CATCGAGGAG AGCCAGGCAC 1260

TGTCCAAGCA AAGCTGCGCT CTCTACCAGA CCTTAGGAGA CTACAAATTA CAAAATCTGT 1320

TCCTTATTGG TTACACGAGG AAAGCCCCTC AGCTGACCTC AGCAGAGCTG ATCGACCTCA 1380

CCGGGAAGAT GGTGAGCATT GCCTCCACGT GCTGCCAGCT CAGCGAGGAG AAATGGTCCG 1440

GCTGTGGTGA GGGAATGGCC GACATTTTCA TTGGACATTT GTGTATAAGG AATGAAGCAA 1500

GCCCTGTGAA CTCTGGTATC AGCCACTGCT GCAACTCTTC GTATTCCAAC AGGAGGCTAT 1560

GCATCACCAG TTTTCTGAGG GATGAAACCT ATGCCCCTCC CCCATTCTCT GAGGATAAAT 1620

TCATCTTCCA CAAGGATCTG TGCCAAGCTC AGGGCAAAGC CCTACAGACC ATGAAACAAG 1680

AGCTTCTCAT TAACCTGGTG AAGCAAAAGC CTGAACTGAC AGAGGAGCAG CTGGCGGCTG 1740

TCACTGCAGA TTTCTCGGGC CTTTTGGAGA AGTGCTGCAA AGCCCAGGAC CAGGAAGTCT 1800

GTTTCACAGA AGAGGGTCCA AAGTTGATTT CCGAAACTCG TGATGCTTTG GGCGTTTAAA 1860

CATCTCCAGA AGGAAGAGTG GACAAAAAAA TGTGTTGACG CTTTGGTGTG AGCCTTTTGG 1920

CTTAACTGTA ACTGCTAGTA CTTTAACCAC ATGGTGAAGA TGTCCATGTG AGATTTCTAT 1980

ACCTTAGGAA TAAAAACTTT TCAACTATT 2009

WHAT IS CLAIMED IS:

1. A method for preventing or for treating cancer in a mammal, the method comprising the step of creating an immune response in the mammal to at least part of the ammino acid sequence of an alphafetoprotein molecule.

2. The method of claim 1, wherein the alphafetoprotein molecule is SEQ ID NO:1.

3. The method of claim 1, wherein the part of the alphafetoprotein molecule is selected from the group consisting of residues 1-9 of SEQ ID NO:1, residues 12-20 of SEQ ID NO:1, residues 158-166 of SEQ ID NO:1, residues 178-186 of SEQ ID NO:1, residues 235-243 of SEQ ID NO:1, residues 287-295 of SEQ ID NO:1, residues 404-412 of SEQ ID NO:1, residues 441-450 of SEQ ID NO:1, residues 492-500 of SEQ ID NO:1, residues 542-550 of SEQ ID NO:1, residues 547-556 of SEQ ID NO:1 and residues 555-563 of SEQ ID NO:1.

4. The method of claim 1, wherein the cancer is hepatocellular carcinoma.

5. The method of claim 1, wherein the mammal is a human.

6. The method of claim 1, wherein the step of creating an immune response comprises administering to the mammal at least one composition including a peptide comprising at least part of the alphafetoprotein amino acid sequence.

7. The method of claim 6, wherein the peptide is selected from the group consisting of residues 1-9 of SEQ ID NO:1, residues 12-20 of SEQ ID NO:1, residues 158-166 of SEQ ID NO:1, residues 178-186 of SEQ ID NO:1, residues 235-243 of SEQ ID NO:1, residues 287-295 of SEQ ID NO:1, residues 404-412 of SEQ ID NO:1, residues 441-450 of SEQ ID NO:1, residues 492-500 of SEQ ID NO:1, residues 542-550 of SEQ ID NO:1, residues 547-556 of SEQ ID NO:1 and residues 555-563 of SEQ ID NO:1.

8. The method of claim 1, wherein the step of creating an immune response comprises administering to the mammal at least one composition including a peptide comprising at least part of the alphafetoprotein amino acid sequence with at least one amino acid substitution.

9. The method of claim 8, wherein the peptide is selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:3.

10. The method of claim 1, wherein the step of creating an immune response comprises administering to the mammal at least one composition including at least part of the cDNA sequence for the alphafetoprotein molecule.

11. The method of claim 10, wherein the alphafetoprotein cDNA is SEQ ID NO:1.

12. The method of claim 1, wherein the step of creating an immune response comprises administering to the mammal at least one composition including immune system cells transduced with a recombinant vector that expresses alphafetoprotein cDNA.

13. The method of claim 12, wherein the immune system cells are dendritic cells.

14. The method of claim 12, wherein the alphafetoprotein cDNA is SEQ ID NO:1.

15. A composition for immunizing a human to prevent or to treat cancer, the composition comprising a peptide selected from the group consisting of residues 1-9 of SEQ ID NO:1, residues 12-20 of SEQ ID NO:1, residues 158-166 of SEQ ID NO:1, residues 178-186 of SEQ ID NO:1, residues 235-243 of SEQ ID NO:1, residues 287-295 of SEQ ID NO:1, residues 404-412 of SEQ ID NO:1, residues 441-450 of SEQ ID NO:1, residues 492-500 of SEQ ID NO:1, residues 542-550 of SEQ ID NO:1, residues 547-556 of SEQ ID NO:1, residues 555-563 of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3.

16. A method of preventing or treating cancer in a human comprising the step of administering to the human the composition of claim 13.

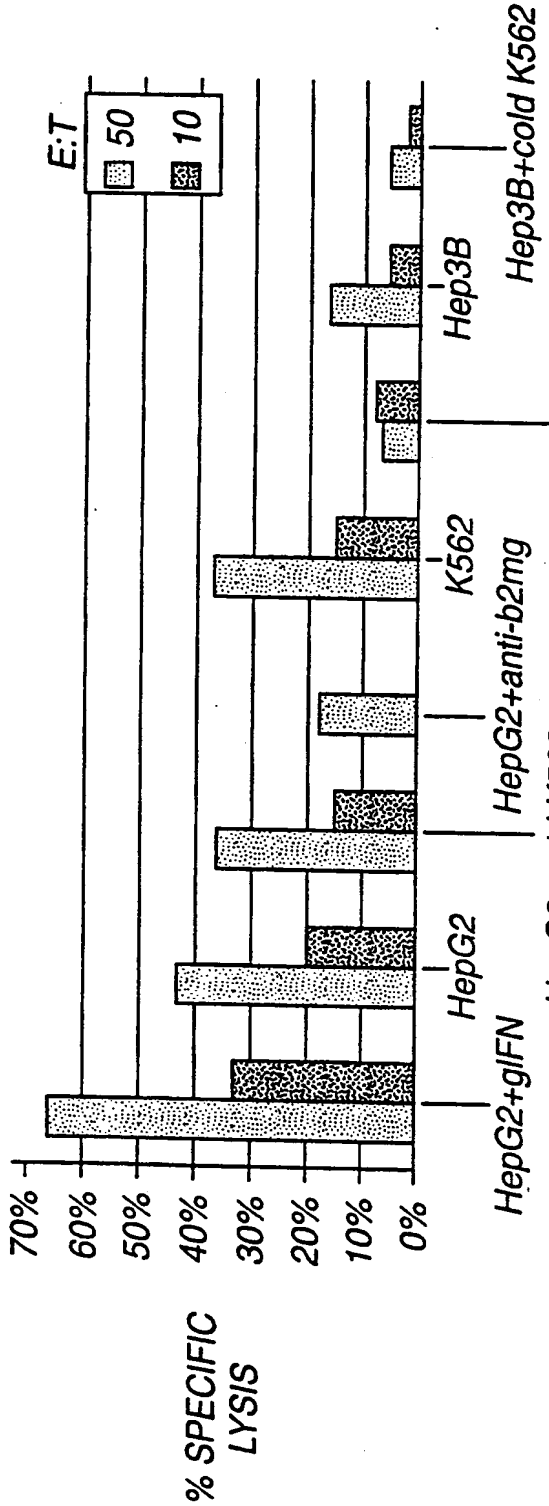

E:T
50
10
% SPECIFIC LYSIS
70%
60%
50%
40%
30%
20%
10%
0%
HepG2+gIFN
HepG2
HepG2+cold K562
HepG2+anti-b2mg
K562
K562+cold K562
Hep3B
Hep3B+cold K562

FIG. 1

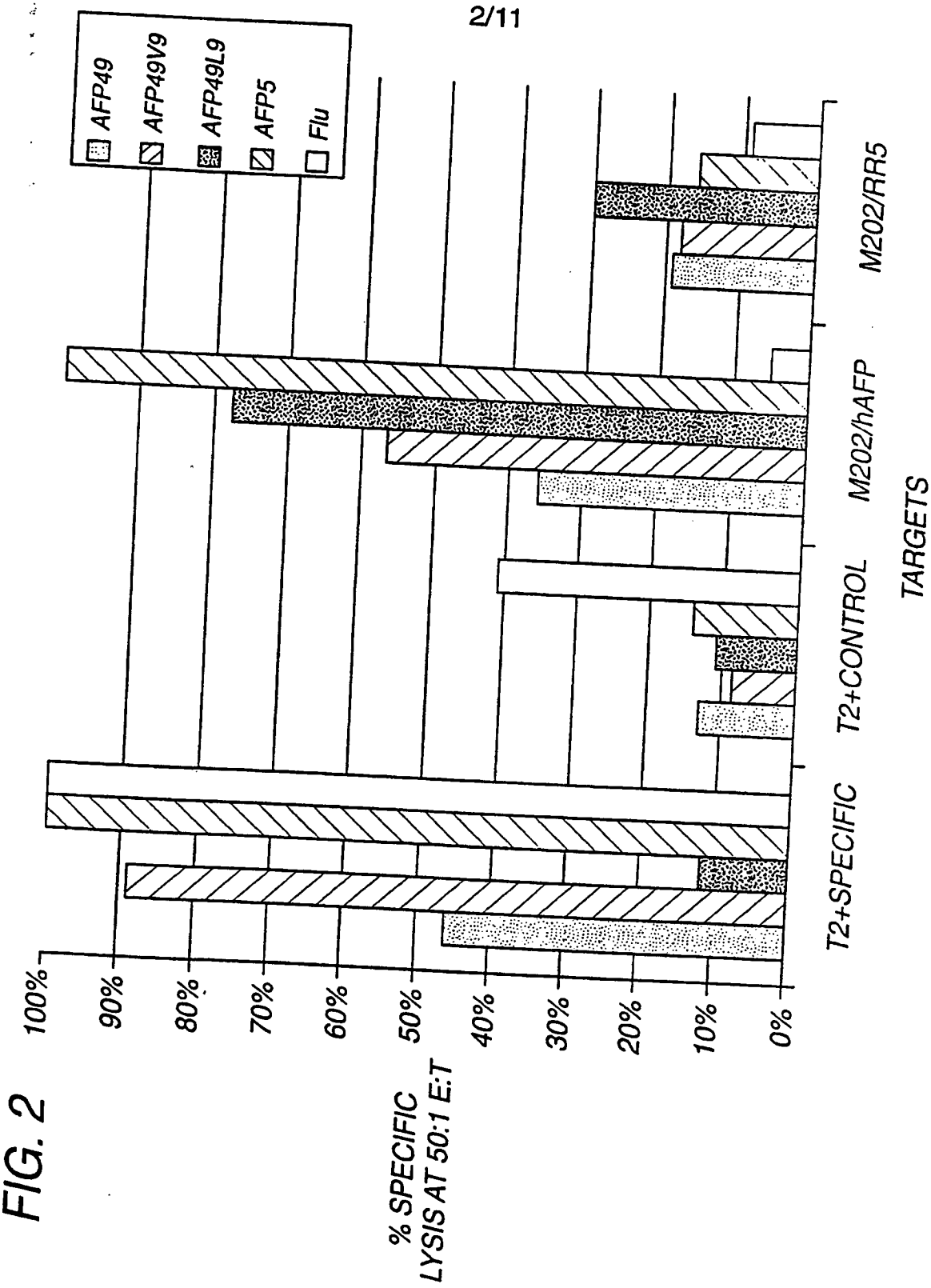

AFP49
AFP49V9
AFP49L9
AFP5
Flu
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% SPECIFIC LYSIS AT 50:1 E:T
T2+SPECIFIC
T2+CONTROL
M202/hAFP
M202/RR5
TARGETS

FIG. 2

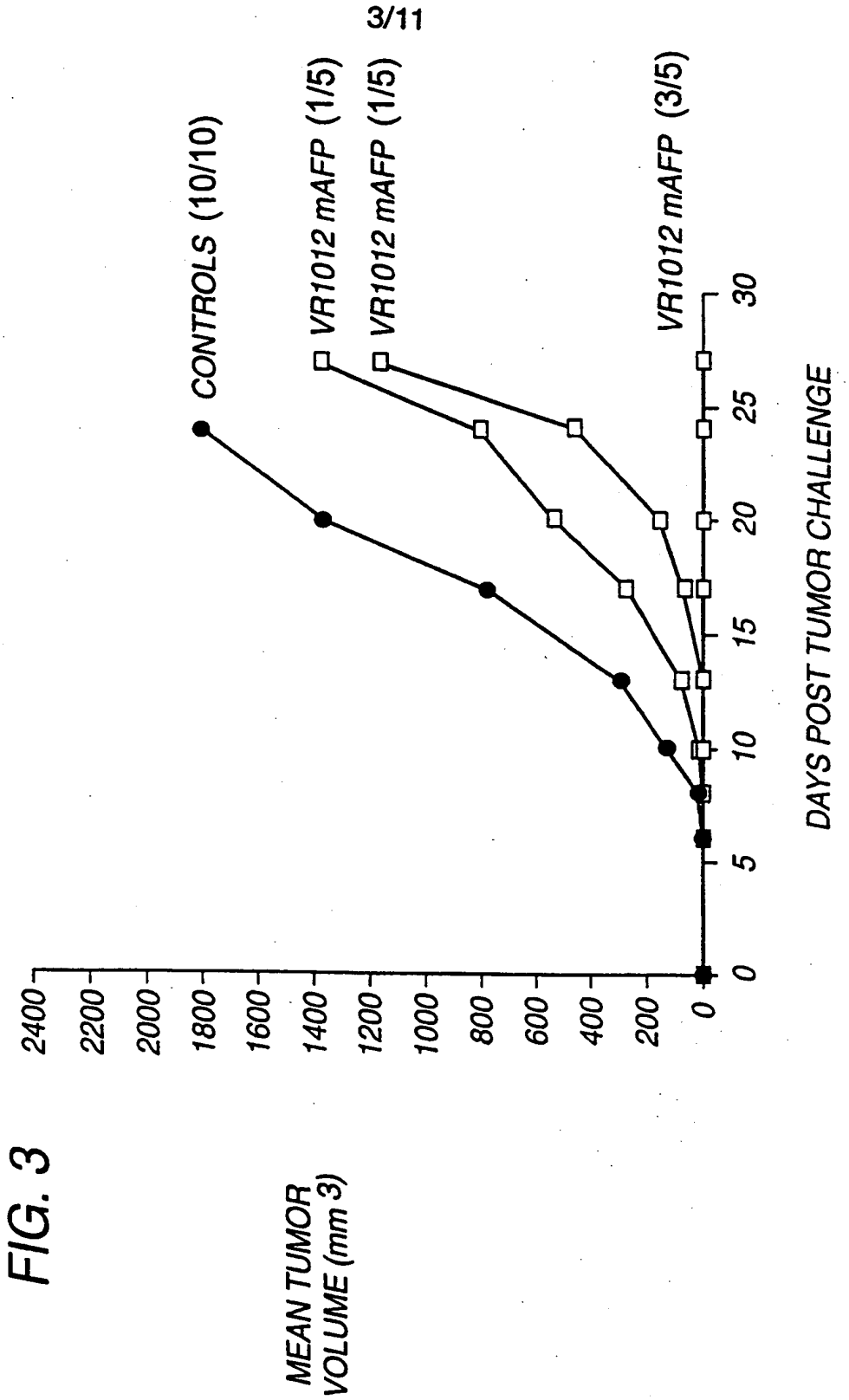

CONTROLS (10/10)
VR1012 mAFP (1/5)
VR1012 mAFP (1/5)
VR1012 mAFP (3/5)
MEAN TUMOR VOLUME (mm 3)
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
5
10
15
20
25
30
DAYS POST TUMOR CHALLENGE

FIG. 3

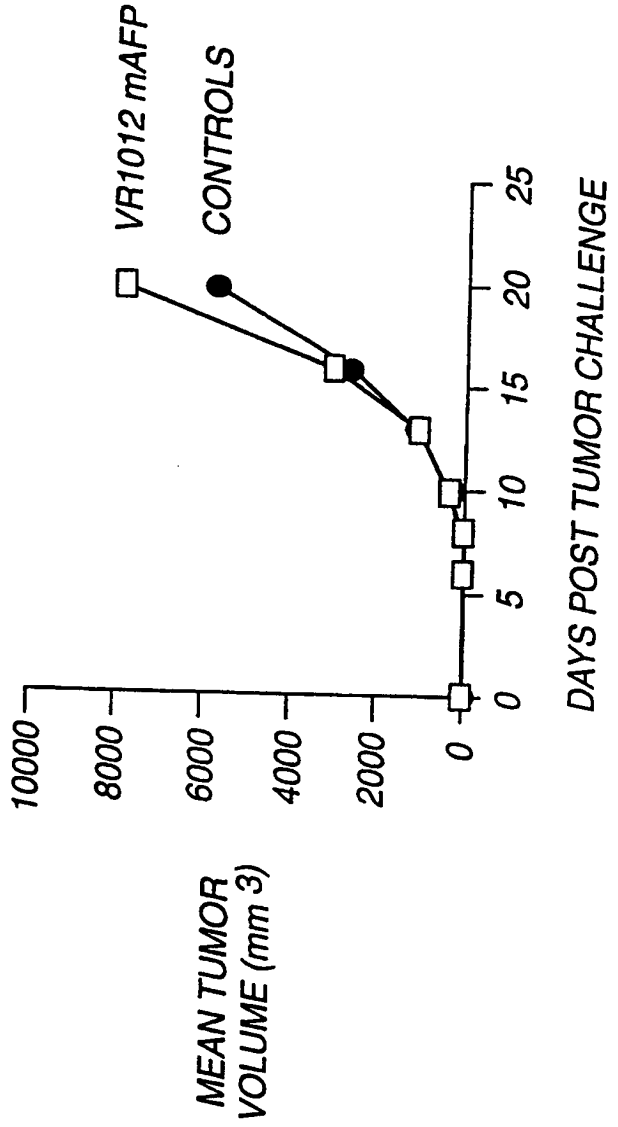

VR1012 mAFP
CONTROLS
10000
8000
6000
4000
2000
0
MEAN TUMOR VOLUME (mm 3)
0
5
10
15
20
25
DAYS POST TUMOR CHALLENGE

FIG. 4

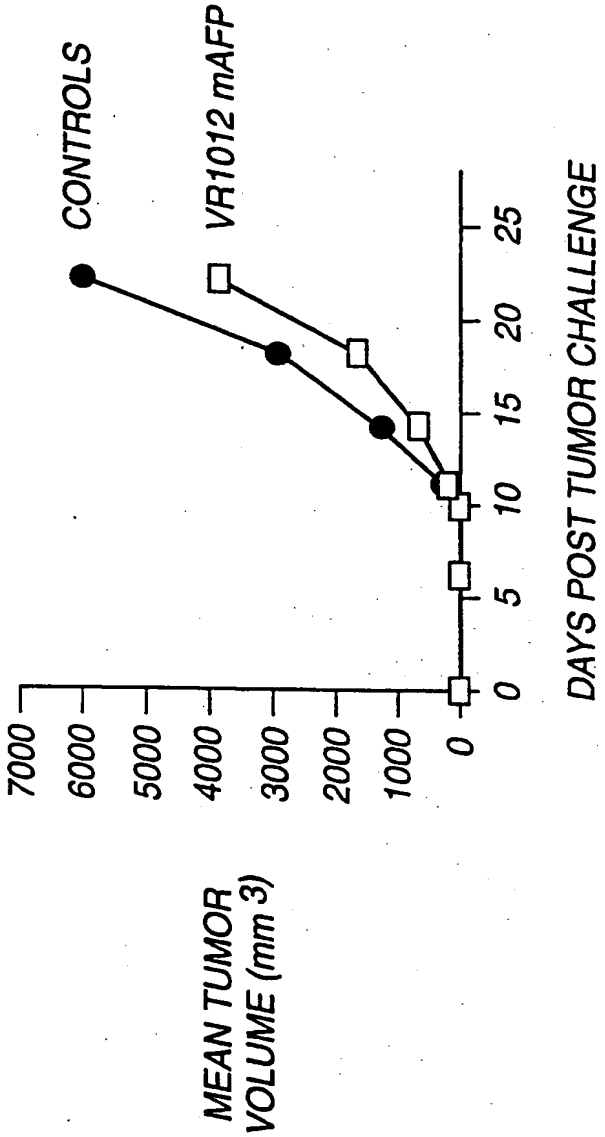

CONTROLS
VR1012 mAFP
7000
6000
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
DAYS POST TUMOR CHALLENGE
MEAN TUMOR VOLUME (mm 3)

FIG. 5

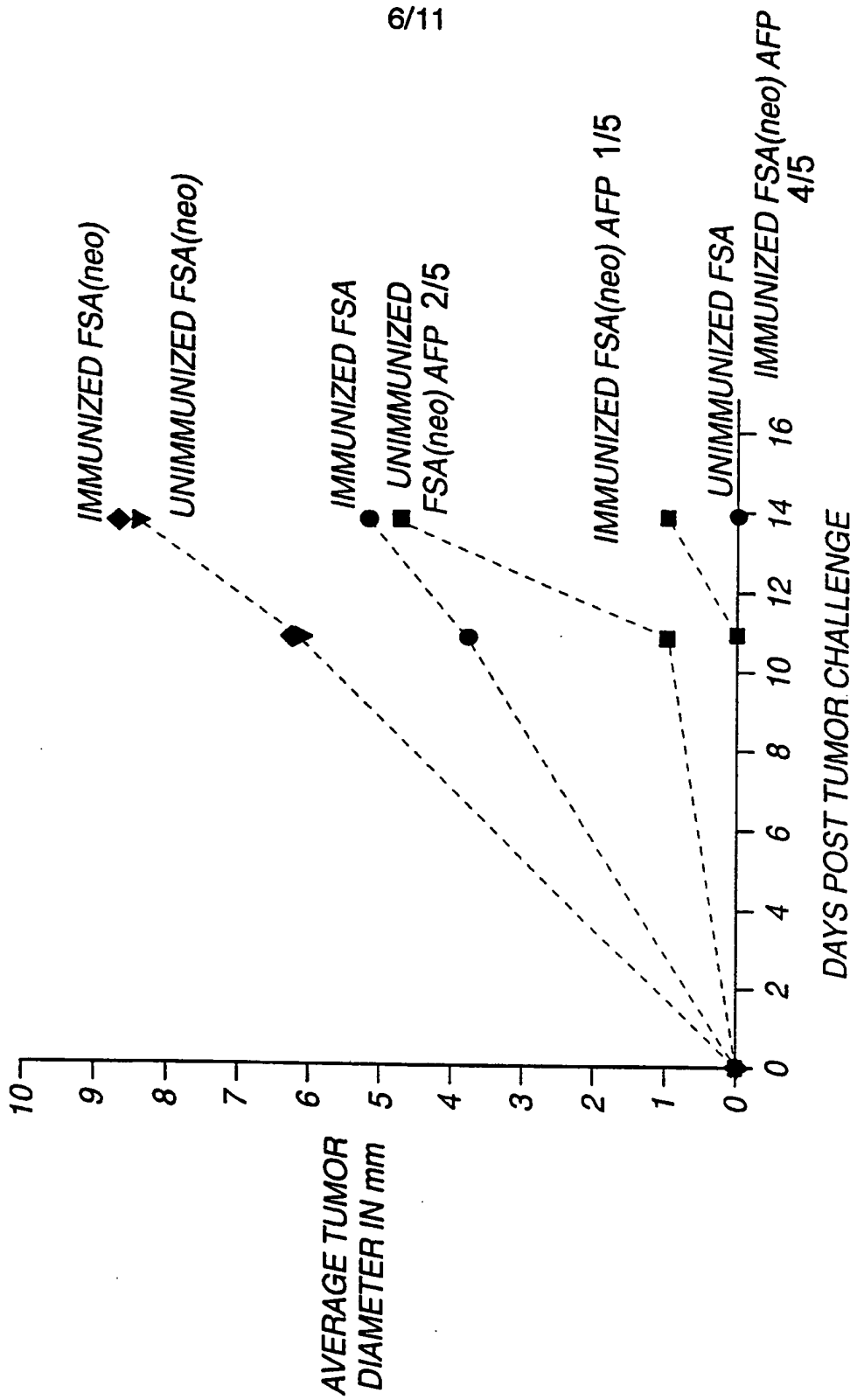

IMMUNIZED FSA(neo)
UNIMMUNIZED FSA(neo)
IMMUNIZED FSA
UNIMMUNIZED FSA(neo) AFP 2/5
IMMUNIZED FSA(neo) AFP 1/5
UNIMMUNIZED FSA
IMMUNIZED FSA(neo) AFP 4/5
AVERAGE TUMOR DIAMETER IN mm
DAYS POST TUMOR CHALLENGE
0
1
2
3
4
5
6
7
8
9
10
0
2
4
6
8
10
12
14
16

FIG. 6

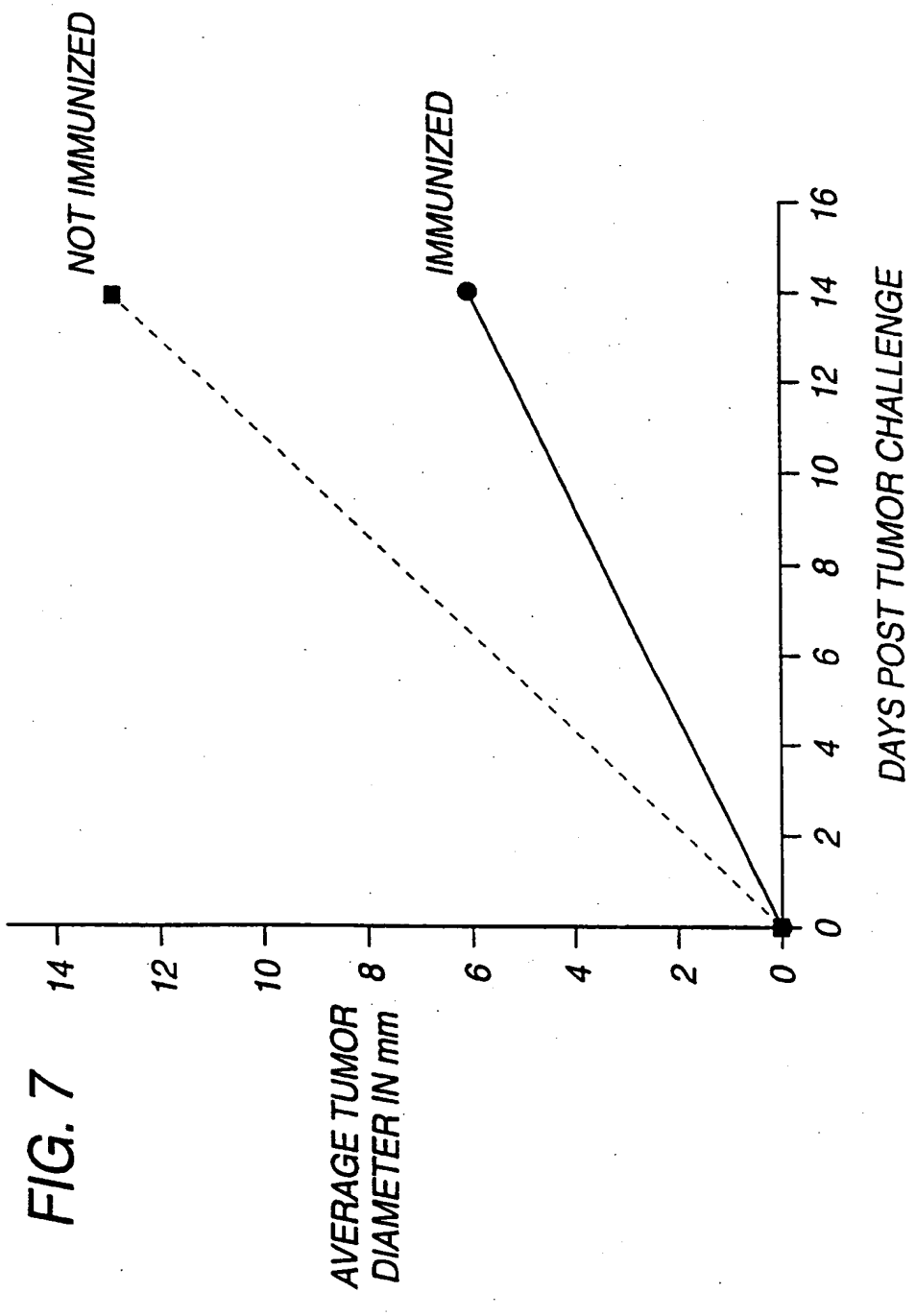

NOT IMMUNIZED
IMMUNIZED
AVERAGE TUMOR DIAMETER IN mm
0
2
4
6
8
10
12
14
DAYS POST TUMOR CHALLENGE
0
2
4
6
8
10
12
14
16

FIG. 7

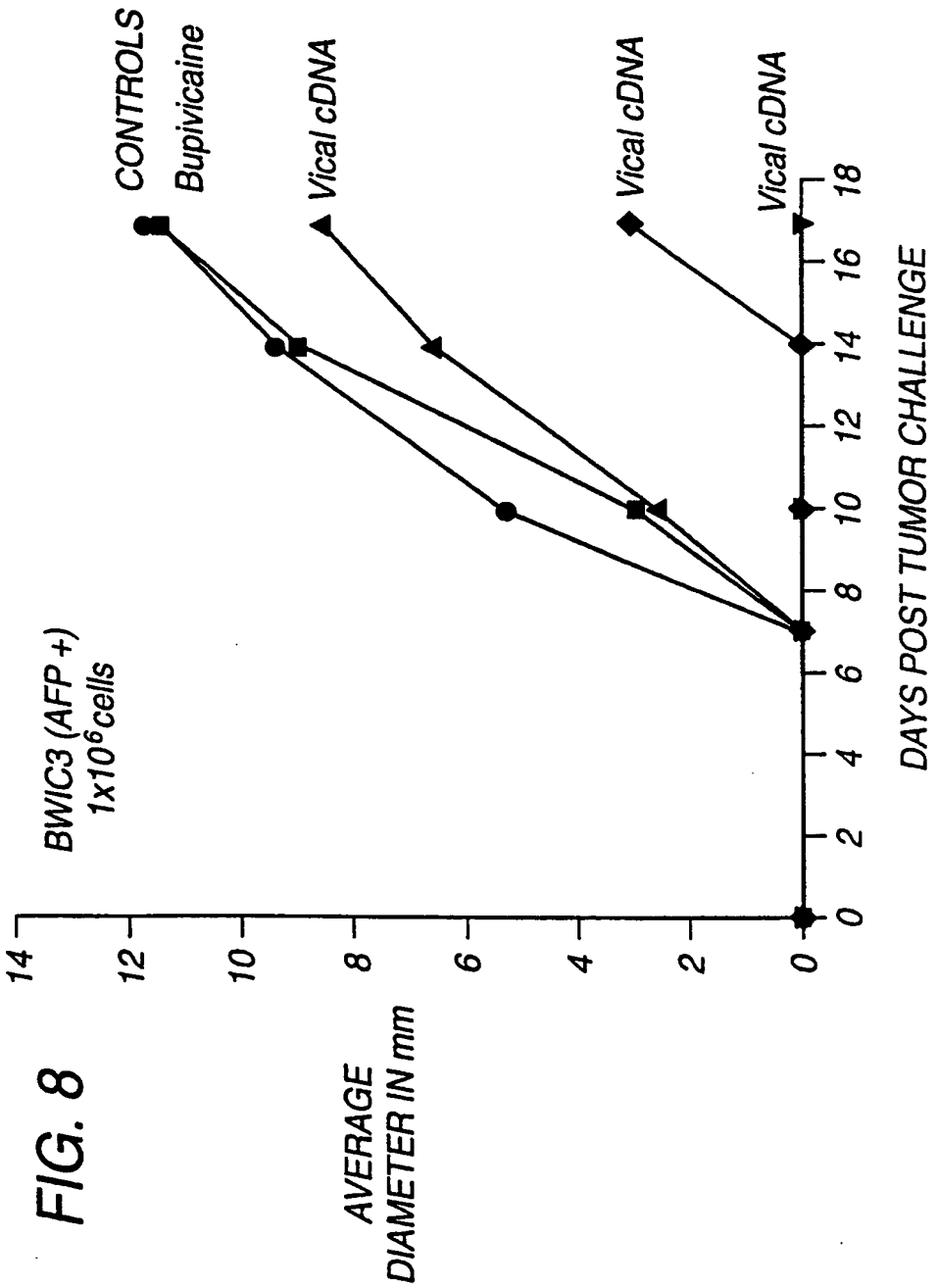

CONTROLS
Bupivicaine
Vical cDNA
Vical cDNA
Vical cDNA
BWIC3 (AFP +)
1x10^6cells
AVERAGE
DIAMETER IN mm
DAYS POST TUMOR CHALLENGE
0
2
4
6
8
10
12
14
16
18

FIG. 8

FIG. 9

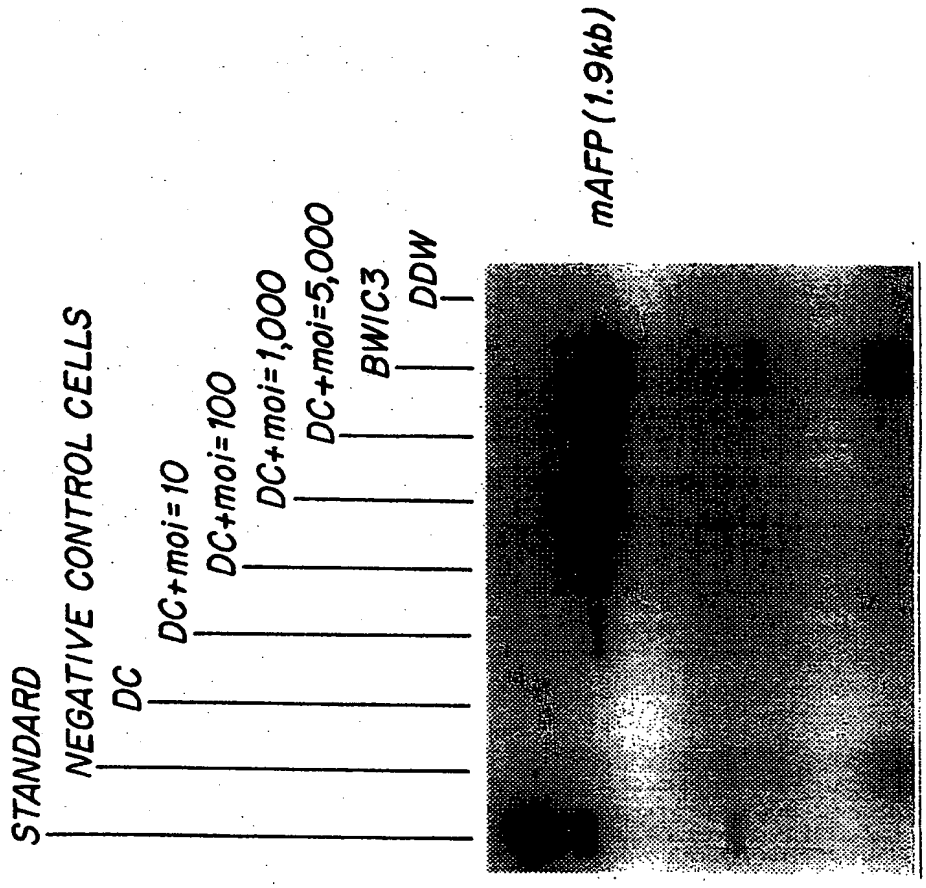
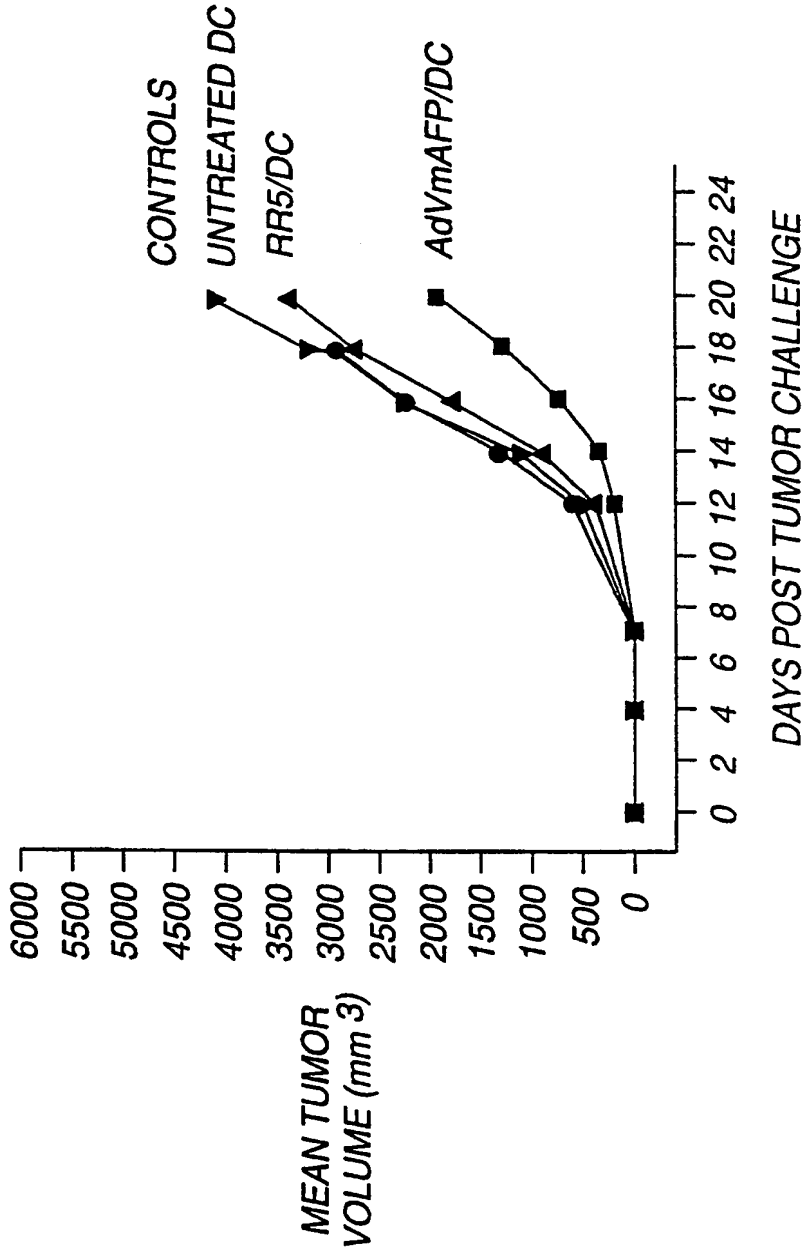

STANDARD
NEGATIVE CONTROL CELLS
DC
DC+moi=10
DC+moi=100
DC+moi=1,000
DC+moi=5,000
BWIC3
DDW
mAFP(1.9kb)

FIG. 10


CONTROLS
UNTREATED DC
RR5/DC
AdVmAFP/DC
MEAN TUMOR VOLUME (mm 3)
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0 2 4 6 8 10 12 14 16 18 20 22 24
DAYS POST TUMOR CHALLENGE

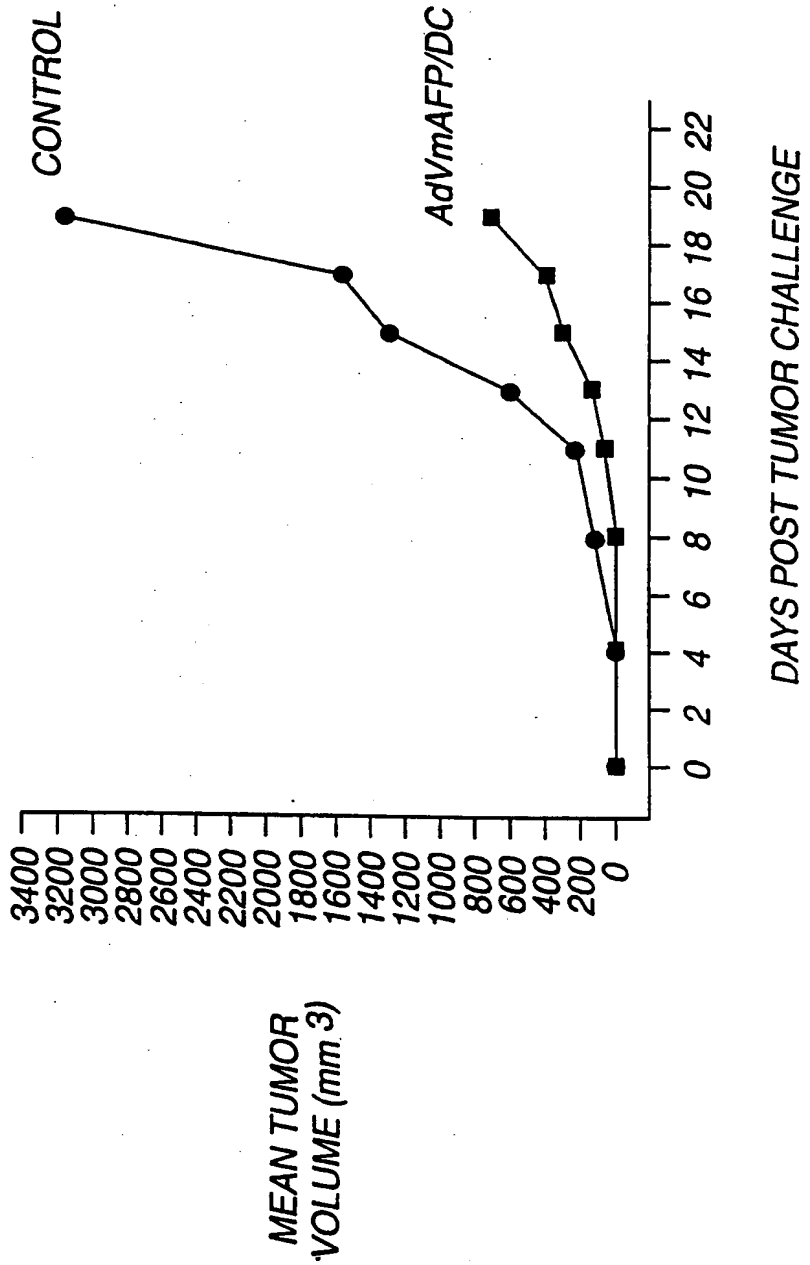

CONTROL
AdVmAFP/DC
MEAN TUMOR VOLUME (mm3)
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0 2 4 6 8 10 12 14 16 18 20 22
DAYS POST TUMOR CHALLENGE

FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/02753

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 5/00, 14/00; A61K, 38/00; A01N 43/04
US CL : 514/2, 12, 44, 885; 530 300, 324, 350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 12, 44, 885; 530 300, 324, 350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	HIRAI et al. Purification of Specific Antibody to Alpha-Foetoprotein and its Immunological Effect on Cancer Cells. Journal of Chromatography, 1981. Vol. 215, pages 195-210, see especially the Summary, page 195.	1-7, 15 ---- 10-14, 16
X -- Y	TAGA. H. The Effect of Active Immunization of Rats with Heterologous Alpha-Fetoprotein upon Hepatocarcinogenesis Induced by 3'-methyl-4-dimethylaminoazobenzene. Gann. April 1983. Vol. 74, pages 248-257, see especially Abstract and page 256.	1-7 and 15 ---- 8-14 and 16

[X] Further documents are listed in the continuation of Box C. [] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 04 MAY 1998	Date of mailing of the international search report 09 JUL 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SUSAN UNGAR Telephone No. (703) 308-0196

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